主論文Web公開版

Title page

- Generation of a replication-competent simian–human immunodeficiency virus, the neutralisation sensitivity of which can be enhanced in the presence of a small molecule CD4 mimic

- The Contents Category for the paper, Short communications

- Short running title: SHIV with conditional neutralisation sensitivity

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- The word count of the summary and the main text,
  Summary, 143 words
  Main text, 2451 words
  Figure legends, 225 words

Summary

Simian-human immunodeficiency virus (SHIV) carrying the envelope from the clade B clinical HIV-1 isolate HIV-1 MNA, designated SHIV MNA, was generated through intracellular homologous recombination. SHIV MNA inherited biological properties from the parental HIV-1, including CCR5 co-receptor preference, resistance to neutralisation by the anti-V3 loop monoclonal antibody KD-247, and loss of resistance in the presence of the CD4-mimic small molecule YYA-021. SHIV MNA showed productive replication in rhesus macaque peripheral blood mononuclear cells.

Experimental infection of a rhesus macaque with SHIV MNA caused a transient but high titre of plasma viral RNA and a moderate antibody response. Immunoglobulin in the plasma at 24 weeks post-infection was capable of neutralising SHIV MNA in the presence but not in the absence of YYA-021.

SHIV MNA could serve a model for development of novel therapeutic interventions based on CD4-mimic-mediated conversion of Env susceptible to antibody neutralisation.

47 **Text**

48 Control of primate lentiviral infection by antibodies directed against viral envelope
49 protein is theoretically feasible. This was confirmed by the successful protection of
50 macaque monkeys from challenge inoculation with simian-human immunodeficiency
51 virus (SHIV) carrying an envelope protein (Env). Env was derived from a laboratory
52 strain of human immunodeficiency virus type 1 (HIV-1) through the passive
53 immunisation of neutralising monoclonal antibodies directed against HIV-1 (Mascola *et*
54 *al.*, 2000; Nishimura *et al.*, 2003). This neutralisation is consistent with the results
55 normally seen in cell culture systems.

56 Clinical isolates of HIV-1, which have not been subjected to extensive passage in
57 T-cell lines, on the other hand, are generally resistant to antibody-mediated
58 neutralisation (Moore *et al.*, 1995). It has been shown that virus in infected individuals
59 is under selective pressure to develop a variety of means to evade attack by neutralising
60 antibodies, including sequence variation, glycosylation, tertiary structural shielding
61 formed by the Env trimer, and the rapid kinetics of conformational changes of Env,
62 which affect fusion between the viral envelope and the plasma membrane of target cells

63 (Kong & Sattentau, 2012). Although four major neutralising epitopes have been
64 identified in the HIV-1 Env; *i.e.* the V1/V2 loop, the glycan-V3 site and CD4-binding
65 site of gp120, and the membrane-proximal external region (MPER) of gp41, few reports
66 of antibodies directed against these epitopes capable of neutralising a broad range of
67 isolates have been published, for reasons that are as yet unclear (Kwong & Mascola,
68 2012). High titres of antibodies directed against the V3 loop are elicited in individuals
69 during the early phase of HIV-1 infection, but these are incapable of neutralising the
70 virus because the epitope in functional Env trimer is likely shielded from the antibody
71 (Davis *et al.*, 2009b). Therefore, it is necessary to develop a means of rendering these
72 epitopes accessible to the antibodies, to make antibody-mediated suppression of HIV-1
73 a valid therapeutic option.

74 It has been reported that neutralisation mediated by antibodies directed against the
75 V3 loop (Lusso *et al.*, 2005) or CD4-induced epitope (CD4i) (Thali *et al.*, 1993) can be
76 enhanced in the presence of soluble CD4 (sCD4). It is known that the interaction of Env
77 with sCD4 drives a conformational change of the viral protein and makes the
78 cryptic/occult epitopes accessible to these antibodies (Wyatt *et al.*, 1998). Small

79 molecules that emulate sCD4 for its interaction and subsequent induction of
80 conformational change of Env may be employed to intensify antibody-mediated
81 interventions against HIV-1 infection. Compounds with the above-mentioned
82 properties; *i.e*. NBD-556 and NBD-557, have been reported previously (Zhao *et al.*,
83 2005). NBD-556 has been shown in cell culture to interact with the CD4-binding pocket
84 to induce a conformational change in gp120 (Madani *et al.*, 2008) and enhance exposure
85 of the Env of primary HIV-1 isolates to neutralising epitopes (Yoshimura *et al.*, 2010).
86 The present study was performed to evaluate small molecule CD4-mimic-based
87 enhancement of antibody-mediated virus neutralisation, in the context of virus infection
88 *in vivo*. The simian-human immunodeficiency virus (SHIV)/macaque monkey model of
89 AIDS is particularly suitable for such studies, as SHIV carries the HIV-1 Env and the
90 neutralisation sensitivity of SHIV is comparable to that of the parental HIV-1 (Shibata
91 & Adachi, 1992).
92 As NBD-556, unlike sCD4, inhibits infection with select HIV-1 isolates (Yoshimura
93 *et al.*, 2010), we generated a new SHIV strain carrying Env, the sensitivity of which to
94 antibody-mediated neutralisation is enhanced in the presence of a CD4 mimic. An

HIV-1 isolate, MNA, previously designated primary isolate HIV-1 Pt.3, was used as the source of Env, as the viral protein has been reported to interact with NBD-556 (Yoshimura *et al.*, 2010). While the virus belongs to a distinct subset of HIV-1 isolates, as mentioned above, it has also been reported to utilise the CCR5 molecule to gain entry into target cells, a property that is shared by the majority of HIV-1 strains (Yoshimura *et al.*, 2010). A monoclonal antibody directed against the tip of the V3 loop (GPGR), KD-247 (Eda *et al.*, 2006), was employed to assess this concept, as HIV-1 MNA was resistant to KD-247-mediated neutralisation, despite carrying the exact epitope sequence in the tip of V3 loop, and was converted to being sensitive to the antibody by NBD-556 in a dose-dependent manner (Yoshimura *et al.*, 2010).

First, we reproduced the results of Yoshimura *et al*. using a neutralisation assay employing TZM-bl cells (Platt *et al.*, 1998), obtained from the NIH AIDS Reagent program (Fig. S1). The virus was resistant to KD-247, as described previously, and required almost 50 μg/mL of the antibody to achieve 50% neutralisation in our assay. The observed resistance was abrogated in the presence of 2 μM of NBD-556. However, 50% neutralisation was achieved in the presence of ~0.1 μg/mL of KD-247,

111 corresponding to 1/500 of the amount of the antibody to achieve the same degree of
112 neutralisation in the absence of the CD4 mimic.

113 With reproduction of the properties of HIV-1 MNA Env, we generated an SHIV
114 strain carrying Env through intracellular homologous recombination, as described
115 previously (Fujita *et al.*, 2013) with minor modifications (Fig. S2). DNA fragments
116 representing the 5' and 3' ends of the SHIV genome (fragments I and II, respectively)
117 were amplified by PCR from the proviral DNA plasmid SHIV KS661. A DNA
118 fragment containing *env* (fragment III) was amplified from complementary DNA
119 (cDNA) of the HIV-1 MNA genome, which was prepared from virus particles
120 (virion-associated RNA) in the culture supernatant of PM1/CCR5 cells (Yusa *et al.*,
121 2005) infected with the virus. The PCR primers used are listed in Table S1. Using a
122 FuGENE HD transfection reagent, lipofection was performed on the C8166-CCR5 cells
123 (Shimizu *et al.*, 2006) to co-transfect them with 0.2 μg of DNA. A cytopathic effect
124 presumably caused by the emerged recombinant virus was observed on day 13
125 post-transfection. The emerged virus, designated SHIV MNA, carried the entire gp120
126 and three quarters of gp41 from HIV-1 MNA Env (Fig. 1a). The rest of Env was from

SHIV KS661, the Env of which was derived from HIV-1 89.6 (Shinohara *et al.*, 1999). The CD4 binding site, and the regions and elements that reportedly interact with NBD-556 (Madani *et al.*, 2008; Yoshimura *et al.*, 2010), are preserved in SHIV MNA Env (Fig. S3). The virus was replication-competent in PM1/CCR5 cells (data not shown).

As HIV-1 MNA was suggested to be a CCR5-utilising virus, we were intrigued whether SHIV MNA inherited the trait from the parental virus. We subjected SHIV MNA and the parental HIV-1 MNA to co-receptor usage assay as described previously (Nishimura *et al.*, 2010), with minor modifications (Fig. S4). As expected, SHIV MNA was shown to utilise CCR5 as an entry co-receptor.

We next assessed the neutralisation profiles of SHIV MNA in comparison with the parental HIV-1 MNA, as described previously (Li *et al.*, 2005; Wei *et al.*, 2002). Both SHIV MNA and HIV-1 MNA showed essentially no neutralisation by KD-247 up to 25 μg/mL and 50% neutralisation was achieved at 50 μg/mL (Fig. 1b). As the CD4 mimic, we employed YYA-021, a compound generated and characterised through studies concerning the structure-activity relationships of small molecules (Narumi *et al.*, 2013;

143 Narumi *et al.*, 2011; Narumi *et al.*, 2010; Yamada *et al.*, 2010). The compound was
144 shown to be slightly less potent but to exhibit substantially lower toxicity than
145 NBD-556, and was therefore a suitable choice for our purposes in future studies in
146 animal models. SHIV MNA was resistant to neutralisation by YYA-021 at all
147 concentrations examined, except 25 and 50 μM, and showed a neutralisation profile
148 almost identical to that of HIV-1 MNA (Fig. 1c). To further characterise the biological
149 properties of SHIV MNA Env, a set of entry assays was conducted (Fig. S5). The *env*
150 genes cloned from SHIV MNA and HIV-1 MNA, were utilised to generate
151 pseudo-typed viruses. These pseudotypes were inoculated into TZM-bl cells in the
152 presence of increasing amounts of NBD-556, YYA-021 or soluble CD4. A control
153 group was derived from another virus preparation pseudotyped with A-MLV Env
154 (Landau *et al.*, 1991). When the efficiency of entry was defined by intracellular
155 luciferase activities, virtually no difference was observed between Envs of SHIV MNA
156 and the parental HIV-1. Thus SHIV MNA Env replicated in C8166-CCR5 cells retained
157 sensitivity to small molecule CD4 mimics and soluble CD4 comparable to that of
158 HIV-1 MNA.

159 We next examined whether the synergistic neutralisation of HIV-1 MNA by
160 KD-247 antibody in the presence of NBD-556 (Yoshimura *et al.*, 2010) would be
161 reproduced when CD4 mimic was substituted by YYA-021. The synergistic
162 neutralisation effect of KD-247 and YYA-021 was reproduced in our experiments (Fig.
163 1d). At 50 μg/mL, KD-247 barely achieved 50% neutralisation of HIV-1 MNA but
164 resulted in 50% neutralisation at < 0.05 μg/mL in the presence of 20 μM of YYA-021.
165 Finally, to examine whether these two agents neutralise SHIV MNA in the same
166 manner as the parental HIV-1, we conducted a neutralisation assay with KD-247 in the
167 presence of increasing amounts of YYA-021 (0, 5, 10, 20 and 40 μM) (Fig. 1e). The
168 neutralisation curve of KD-247 against SHIV MNA showed an upward shift as the
169 concentration of YYA-021 increased (Fig. 1e), similar to the observations with HIV-1
170 (Fig. 1d), indicating augmentation of neutralisation, and complete neutralisation of both
171 viruses was achieved at 20 μM YYA-021 (Fig. 1d and e). Based on these results, we
172 concluded that the neutralisation profile of SHIV MNA was comparable to that of
173 HIV-1 MNA.
174 Reproduction of the neutralisation characteristics of HIV-1 MNA in the newly

175 generated SHIV prompted us to assess the ability of SHIV MNA to replicate in monkey
176 cells. SHIV MNA, along with SIV239 and SHIV KS661, were normalised with
177 infectious titres and inoculated into rhesus macaque peripheral blood mononuclear cell
178 (PBMC) preparations from four animals, as described previously (Fujita *et al.*, 2013)
179 (Fig. 2a). SHIV KS661, a CXCR4-utilising virus, replicated to the highest titres of all
180 the viruses in all PBMC preparations. Compared to SHIV KS661, SIV239 replicated to
181 lower titres. Under these experimental conditions, SHIV MNA showed productive
182 replication in the cells with similar replication kinetics and peak titres to SIV239. Based
183 on these results, we concluded that SHIV MNA was replication-competent in primary
184 monkey lymphocytes.

185 Productive replication of SHIV MNA in monkey PBMC justified experimental
186 infection of the virus *in vivo*. We inoculated 1.75×10^5 $TCID_{50}$ of SHIV MNA
187 intravenously into a rhesus macaque and monitored plasma viral RNA burden and
188 circulating $CD4^+$ T-lymphocyte levels. Plasma viral RNA burden reached a peak of
189 5.6×10^6 copies/mL at 1 week post-infection (wpi), and declined rapidly thereafter
190 reaching low levels of detection at 7 wpi (around 2.8×10^2 copies/mL). Circulating $CD4^+$

191 T-cell numbers showed a transient decrease around 1 wpi, rebounded around 3 wpi and
192 stabilised around 70% of the pre-infection level from 4 wpi. During the period of
193 observation, the animal developed no obvious clinical manifestations related to
194 lentivirus infection.

195 As SHIV MNA replicated *in vivo* without depleting helper T-cells, it was expected
196 that the animal mounted an anti-viral immune reaction. The production of antibody
197 directed against Env was assessed by western immunoblotting, as described previously
198 (Igarashi *et al.*, 1999). Purified Env protein (Advanced Biotechnologies Inc. Md.
199 U.S.A.) was used as the antigen (Fig. 3a). Anti-Env antibody was detected at 3 wpi and
200 the level of antibody—judged by the intensity of the band—increased gradually with
201 time.

202 We next examined whether the animal generated neutralising antibodies against
203 SHIV MNA. Because plasma samples from this specimen exhibited high background
204 activity, immunoglobulin G (IgG) was purified from these samples collected on day 0
205 and in week 24 post-infection using protein G spin columns (GE healthcare Japan.
206 Tokyo. Japan). While the IgG from day 0 exhibited no neutralising activity (Fig. 3b), as

207 expected, the immunoglobulin collected at 24 wpi neutralised SHIV MNA, although a
208 concentration > 100 μg/mL was required to suppress replication of 100 $TCID_{50}$ of the
209 input virus (Fig. 3c).

210 We examined whether the observed marginal neutralisation by the antibody could be
211 enhanced by the presence of YYA-021. Upon addition of YYA-021 in the assay system,
212 SHIV MNA became sensitive to IgG obtained at 24 wpi (Fig. 3c), while no
213 enhancement was identified from day 0 (Fig. 3b).

214 In this study, we generated a replication-competent SHIV MNA strain carrying an
215 Env resistant to the monoclonal neutralising antibody KD-247 but conditionally
216 sensitive in the presence of the CD4 mimic YYA-021. As the observed neutralisation
217 characteristics were identical to those of HIV-1 MNA, which contributed the majority
218 of the Env sequence to the chimera, the utility of the CD4 mimic as a means of
219 enhancing antibody-mediated virus neutralisation should be assessed in the context of
220 infection *in vivo*. This concept could be tested during the acute phase of SHIV MNA
221 infection, during which the virus undergoes substantial replication. To examine the
222 feasibility of CD4-mimic-mediated enhancement of virus neutralisation in the context

223 of chronic infection, the conditions under which this type of intervention should be
224 applied to HIV-1-infected patients in a clinical setting, the virus must be modified to
225 sustain productive replication for a longer period. SHIV MNA in the present form does
226 not fulfil this requirement. It is possible that animal-to-animal passage could increase
227 the fitness of the virus in monkeys.

228 This study demonstrated that a CD4 mimic could modulate viral Env protein to be
229 more susceptible to neutralisation by less potent antibodies generated in the context of
230 infection. During the early phase of infection, patients mount high titres of
231 non-neutralising antibodies directed against the V3 loop (Davis *et al.*, 2009a). Patients
232 with HIV-1 clade C generate anti-Env antibodies, including anti-CD4i antibodies, with
233 poor neutralising activity against recent infection (Gray *et al.*, 2007). It is possible that
234 the CD4 mimic YYA-021 causes a conformational change in SHIV MNA Env, which
235 renders sequestered epitope(s) accessible to potentially neutralising IgG, such as the V3
236 loop and CD4i.

237 The current study extended the previous study by Yoshimura *et al*. and used HIV-1
238 MNA belonging to clade B to generate a new SHIV strain carrying Env. The

239 neutralisation sensitivity of this strain is characteristically augmented in the presence of
240 a small molecule CD4 mimic. Similar observations by Decker *et al*. show that
241 infections of a wide range of HIV-1 strains of multiple clades or circulating
242 recombinant forms elicits high titres of anti-CD4i antibodies, These anti-CD4i
243 antibodies neutralise viruses as divergent as HIV-2 in the presence of soluble CD4
244 (Decker *et al.*, 2005). Taking these observations into account, small molecule CD4
245 mimics such as YYA-021 could potentially enhance the neutralisation activity of the
246 antibodies directed against autologous viruses belonging not only to clade B but also to
247 multiple HIV-1 strains of various clades, and possibly even HIV-2. Our results pave the
248 way for a novel therapeutic intervention based on administration of CD4 mimics to
249 patients with HIV to facilitate control of the virus by their own antibodies.
250

Acknowledgements

Thanks should be given to: Drs. Julie Strizki and Paul Zavodny of the Schering-Plough Research Institute, Kenilworth, NJ. U.S.A. for providing AD101; the NIH AIDS Research & Reference Reagent Program for providing TZM-bl cells, SV-A-MLV-env, 4G10 and soluble CD4; the Chemo-Sero-Therapeutic Research Institute (Kaketsuken) for providing MAb KD-247; former and current members of the Igarashi laboratory for discussion and support. This work was supported by a Research on HIV/AIDS grant (H22-AIDS Research-007 and H24-AIDS Research-008) from The Ministry of Health, Labor and Welfare of Japan and by a Grant-in-Aid for Scientific Research (B) (23300156) from the Japan Society for the Promotion of Science.

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406 **Figure legends**

407 Figure 1. Genomic organisation (a) and neutralisation sensitivity (b – e) of SHIV

408 MNA.

409 (a) Grey boxes represent genes derived from SIV239, open boxes those from HIV-1

410 89.6 and filled dark grey boxes those from HIV-1 MNA. (b – e) Percentage

411 neutralisation was calculated as follows: % neutralisation = 100 × {1 − (RLU.N −

412 RLU.B)/(RLU.V − RLU.B)}. RLU, relative luciferase units; RLU.N, RLU in wells with

413 cells, virus and KD-247 and/or YYA-021; RLU.V, RLU in wells with cells and virus;

414 RLU.B, RLU in wells with cells.

415

416 Figure 2. Replication of SHIV MNA in rhesus macaque PBMCs (a) and *in vivo* (b).

417 (a) Multiplicity of infection was adjusted to 0.01 ($TCID_{50}$/cell). (b) Experimental

418 infection of a rhesus macaque with SHIV MNA. SHIV MNA (1.75×10^5 $TCID_{50}$) was

419 intravenously inoculated into a rhesus macaque, and the plasma viral RNA burden

420 (filled circles) and circulating $CD4^+$ T-lymphocytes (open triangles) were monitored.

421

422 Figure 3. Antibody induced against SHIV MNA.

(a) The anti-HIV-1 gp120 antibody response was assessed by immunoblotting with plasma samples collected at the indicated times. An anti-HIV-1 V3 monoclonal antibody 4G10 ascites (1:100) (von Brunn *et al.*, 1993), obtained from the NIH AIDS Reagent program, was used as a positive control (lane anti-V3). (b and c) Neutralisation of SHIV MNA with IgG purified from plasma of the infected rhesus macaque (day 0 and 24 wpi) with/without YYA-021 (20 μM).

Table S1. PCR primers.

Primer	Sequence	Position (nt†)
Fragment I		
SIVU3Not-F	5'-atgcggccgctggaagggatttattacagtgcaag-3'	1 – 25*
Preenv-R	5'-aaagagcagaagacgagtggcaa-3'	6204 – 6226#
Fragment II		
SHenv5.5F	5'-tcataatgatagtaggaggc-3'	8278 – 8297#
SIVU5Eco-R	5'-tgcagaattctgctagggattttcctgcttcggtt-3'	10255 – 10279*
Fragment III		
HIV-1vpr-F	5'-agatggaacaagccccagaaga-3'	5557 – 5578#
SHenv6R	5'-gctgaagaggcacaggctccgc-3'	8525 – 8504#

†, Nucleotide positions of PCR primers were numbered relative to the SIV239 (*, GenBank Accession No. M33262) or HXB2 (#, GenBank Accession No. K03455) genome sequences.

Figure S1. Enhanced neutralisation of HIV-1 MNA by KD-247 in the presence of NBD-556.

100 $TCID_{50}$ of HIV-1 MNA was pre-incubated with increasing amounts of KD-247 with/without 2 μM of NBD-556 at 37°C for 60 min, followed by inoculation into 5×10^3 TZM-bl cells. The cells were lysed at 48 h post-infection and luciferase activity was measured. The percentage of neutralisation was measured as RLU reduction relative to virus control wells.

Figure S2. Genomic organisation of SHIV KS661 and HIV-1 MNA and PCR fragments employed for preparation of DNA fragments for generation of SHIV MNA.

Colour-coded boxes represent genes derived from the following viruses: grey boxes, SIV239; open boxes, HIV-1 89.6; grey boxes, HIV-1 MNA. SHIV KS661 carries *tat*, *rev*, *vpu,* and *env* genes from subtype B HIV-1 89.6. Broad lines represent PCR fragments that were amplified using the primers indicated by arrows (A – F).

Figure S3. Deduced amino acid sequence alignment of Env from HIV-1 MNA, SHIV MNA, and SHIV KS661.

(*) =Amino acids that form part of the CD4 binding site.

(†) = Regions/elements that are reported to interact with NBD-556 (Madani *et al.*, 2008; Yoshimura *et al.*, 2010).

Parts of SHIV MNA Env, that were putatively derived from HIV-1 MNA or SHIV KS661 are respectively color-coded as grey or black.

Figure S4. Co-receptor preference of SHIV MNA.

SHIV MNA, along with controls for CCR5-tropic (SIV239) and CXCR4-tropic (HIV-1 NL4-3) and the parental HIV-1 MNA, were inoculated into TZM-bl cells in the presence of increasing amounts of AD101 (Trkola *et al.*, 2002), provided by Dr. J. Strizki, Schering Plough Research Institute, Kenilworth, NJ, and/or AMD3100 (Sigma-Aldrich, St. Louis, MO) (Donzella *et al.*, 1998).

Figure S5. Sensitivity of Env proteins from HIV-1 MNA and SHIV MNA to soluble CD4 and small-molecule CD4 mimics.

Pseudotyped viruses carrying Env from SHIV MNA or HIV-1 MNA were normalised by infectious titre at 100 $TCID_{50}$ and inoculated to TZM-bl cells in the presence of increasing amounts of NBD-556, YYA-021 or soluble CD4. A pseudotyped virus bearing A-MLV Env is acting as the negative control.

Figure S1

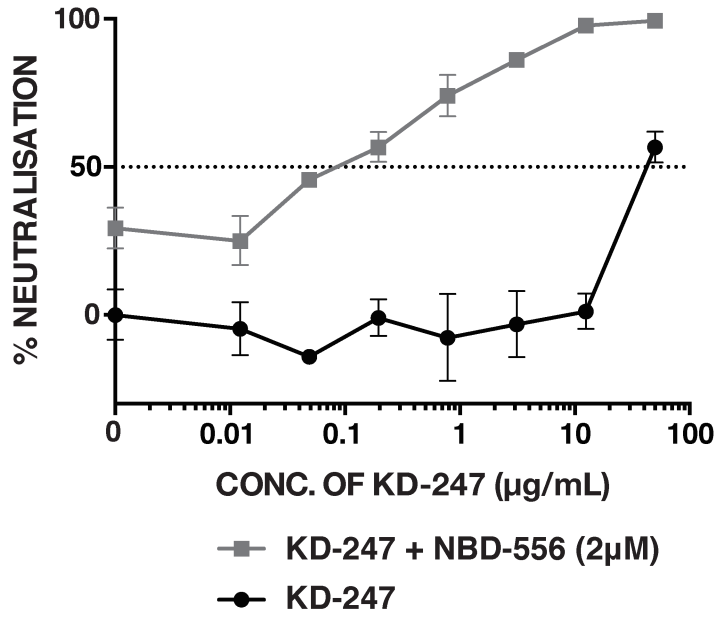

100
50
0
% NEUTRALISATION
0
0.01
0.1
1
10
100
CONC. OF KD-247 (µg/mL)
KD-247 + NBD-556 (2µM)
KD-247

Figure S2

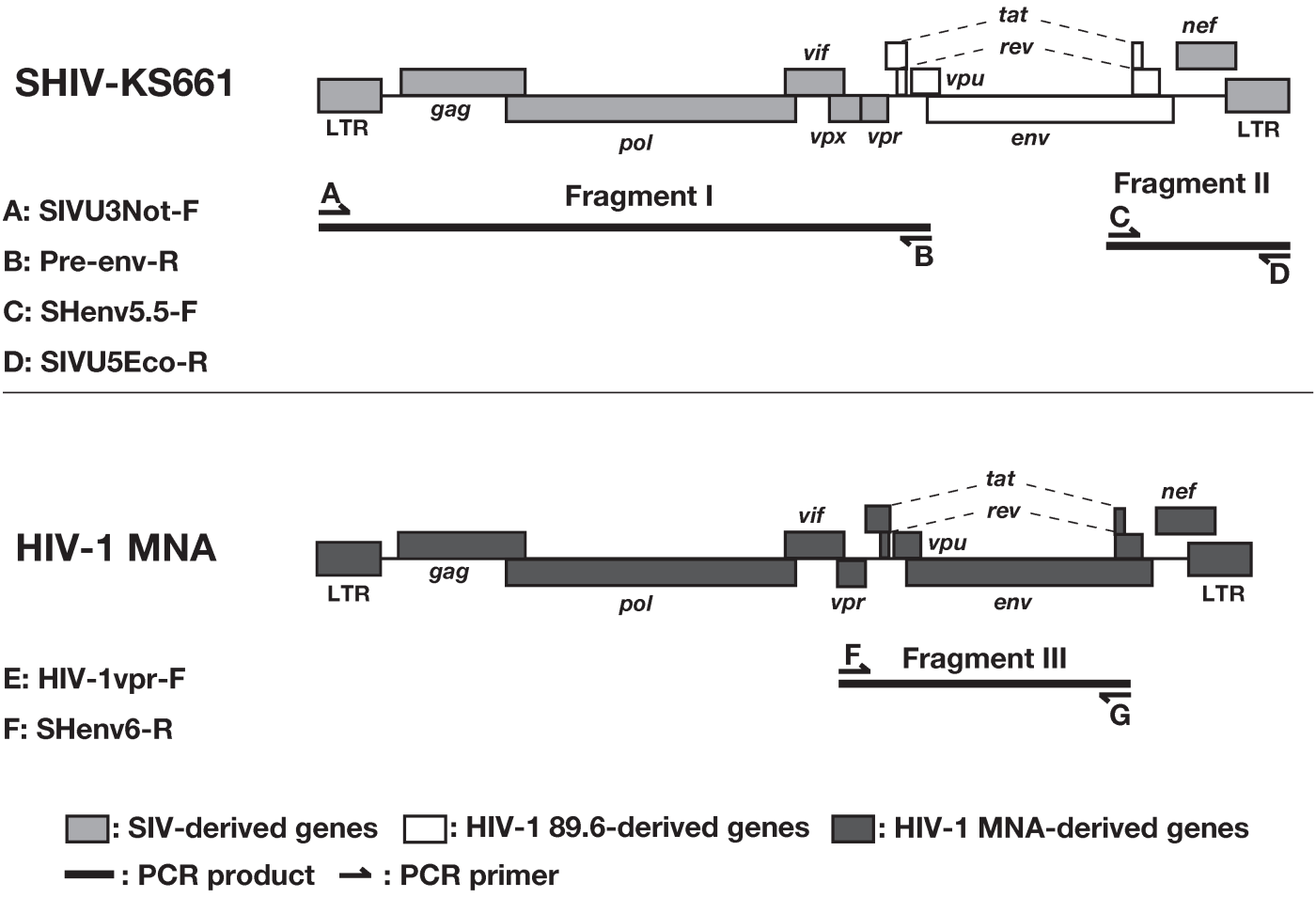

SHIV-KS661
tat
rev
nef
vif
vpu
LTR
gag
pol
vpx
vpr
env
LTR
A
Fragment I
B
Fragment II
C
D
A: SIVU3Not-F
B: Pre-env-R
C: SHenv5.5-F
D: SIVU5Eco-R
HIV-1 MNA
tat
rev
nef
vif
vpu
LTR
gag
pol
vpr
env
LTR
F
Fragment III
G
E: HIV-1vpr-F
F: SHenv6-R
: SIV-derived genes
: HIV-1 89.6-derived genes
: HIV-1 MNA-derived genes
: PCR product
: PCR primer

Figure S3

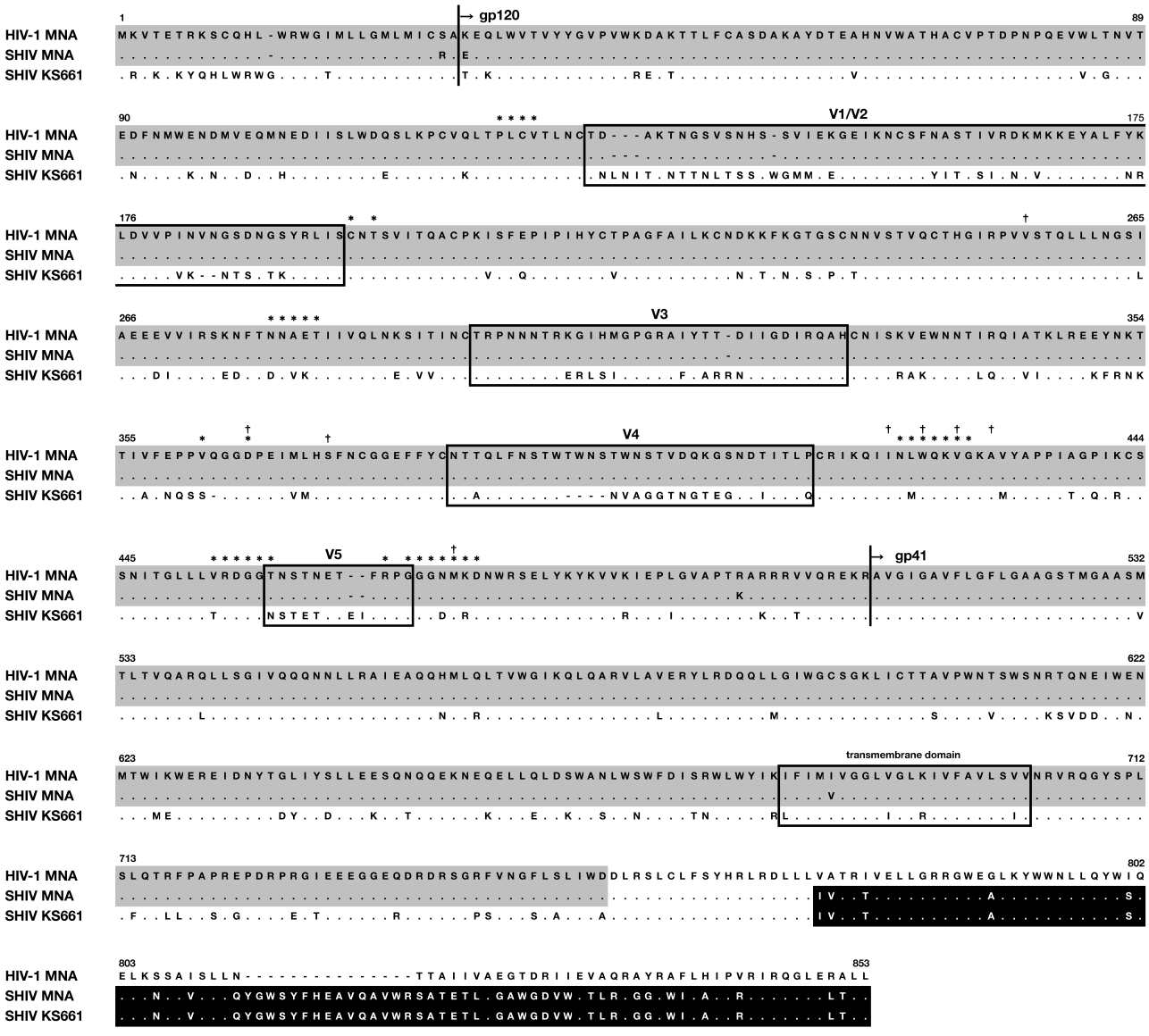

gp120
V1/V2
V3
V4
V5
gp41
transmembrane domain
HIV-1 MNA
SHIV MNA
SHIV KS661

Figure S4

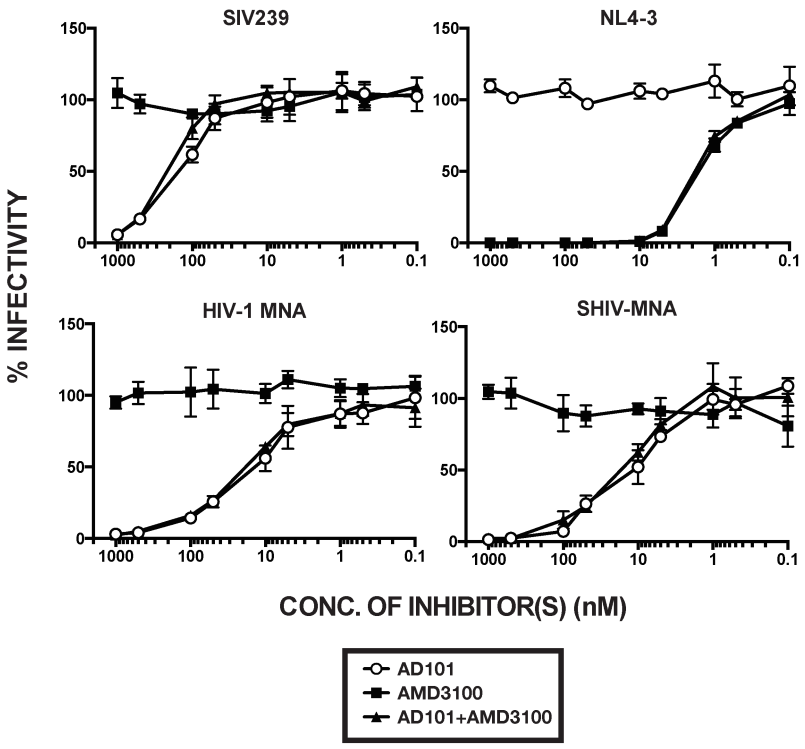
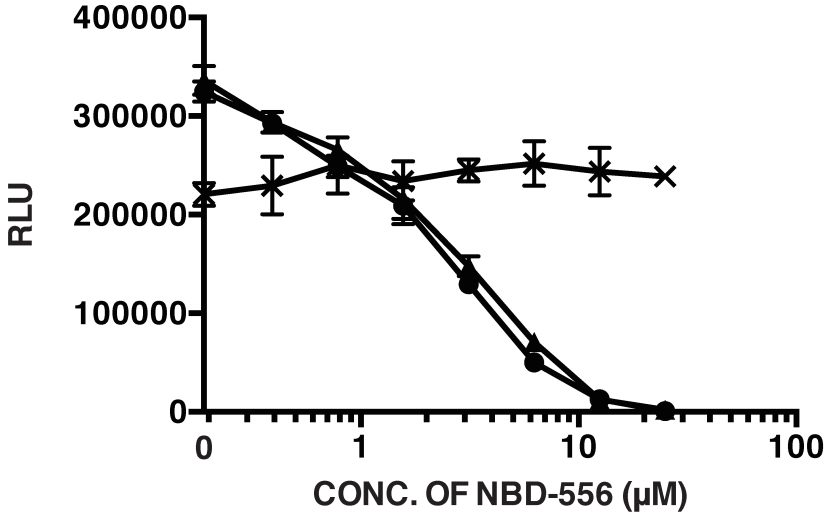
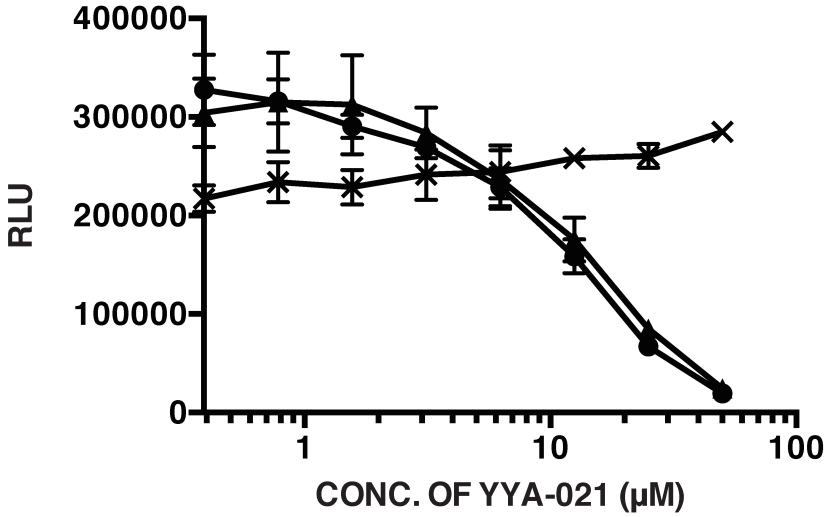
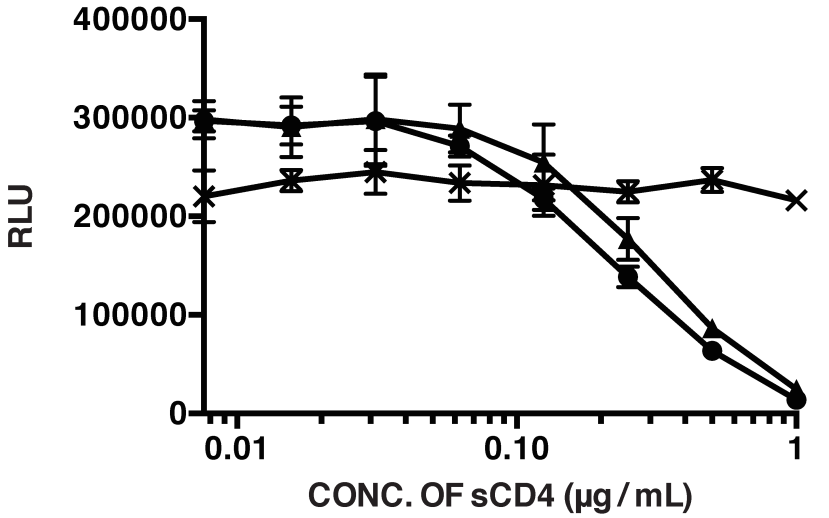
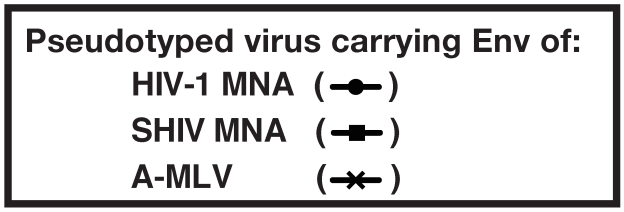

SIV239
NL4-3
HIV-1 MNA
SHIV-MNA
% INFECTIVITY
150
100
50
0
1000
100
10
1
0.1
CONC. OF INHIBITOR(S) (nM)
AD101
AMD3100
AD101+AMD3100

Figure S5


400000
300000
200000
100000
0
RLU
0
1
10
100
CONC. OF NBD-556 (μM)


400000
300000
200000
100000
0
RLU
1
10
100
CONC. OF YYA-021 (μM)


400000
300000
200000
100000
0
RLU
0.01
0.10
1
CONC. OF sCD4 (μg / mL)


Pseudotyped virus carrying Env of:
HIV-1 MNA
SHIV MNA
A-MLV